

REMARKS

This submission is in response to the Final Office Action dated February 22, 2002. Claims 1-106, 126-129, 132, and 137-145 have been canceled, without prejudice or disclaimer. Claims 146-159, 161, 163-164, and 166-167, and 169 have been amended. New claims 170-189 have been added. Claims 146-189 are pending. Reconsideration of the above identified application, in view of the above amendments and the following remarks, is respectfully requested.

As proposed by the Examiner, claims 146, 148, 150, 152, and 155, as well as claim 167, have been amended to recite that the oxygenase variant is functional, *i.e.*, has oxygenase activity. This amendment is supported, *e.g.*, at page 53, line 11, to page 54, line 6).

Claims 146, 148, 150, 152, 156-159, 161, 164, and 167 have been amended to recite that the cytochrome P450 oxygenase variant has at least 90% sequence identity to SEQ ID NO:2, *i.e.*, the sequence of wild-type cytochrome P450 oxygenase from *P. putida*. This is supported by the specification as filed, *e.g.*, at page 21, lines 7-15, and by Example 8, pages 51-57 (see, in particular, page 55, lines 20-28), as well as by SEQ ID NOS: 11-13.

Claims 146, 148, 150, 153, and 154 have been amended to recite specific types of amino acids that are mutated to provide cytochrome P450 oxygenase variants of the invention. This amendment is supported, *e.g.*, by Example 8, pages 51-57 (see, in particular, page 55, lines 20-28).

Claims 147, 149, and 151 have been amended to recite that the amino acid in the claim is the mutant amino acid, as opposed to the mutated amino acid, *i.e.*, the amino acid that is part of the wild-type enzyme.

Claim 154 has been amended to depend from claim 152 and to recite a variant comprising at least one mutation selected from arginine or histidine at position 331 and isoleucine, methionine, or valine at position 280 of SEQ ID NO:2. This amendment is supported by the specification at, *e.g.*, page 21, lines 1-7.

Claim 155 has been amended to recite hybridization under conditions of high stringency. This amendment is supported, *e.g.*, at page 22, lines 5-8 of the specification.

Claims 156-159 have also been amended to recite "a cytochrome P450 oxygenase variant" instead of "an evolved cytochrome P450 oxygenase variant". This amendment is supported throughout the specification, *e.g.*, at page 15, lines 2-7.

Claims 161 and 164 have also been amended to recite a step of selecting any test enzyme having a specific activity/stability and at least 90% sequence identity to SEQ ID NO:2, and claim 167 has been amended to recite selecting any test enzyme having the recited mutations. These amendments are supported, *e.g.*, by Example 8 of the specification.

Claims 163, 166, and 169 have been amended to recite "NOVOZYME® 502" instead of "peroxidase Novozyme® 502".

New claims 170-189, directed to cytochrome P450 variants of SEQ ID NO:2 comprising at least one mutation, or having improved oxygenase activity in the presence of a peroxide oxygen donor, are fully supported by the specification as filed, see, *e.g.*, page 16, lines 3-8; Example 2, pages 35-42 (in particular, page 38, lines 6-8 and page 39, lines 27-32); Example 8, pages 51-57 (in particular, page 51, lines 22-24; page 54, lines 25-34; and page 55, lines 20-28) and SEQ ID NOS: 11-13.

No new matter has been added by way of this amendment.

Rejections Under 35 U.S.C. 112, 2nd Paragraph

Claims 146-153 and 160 have been rejected as allegedly indefinite for not reciting that the cytochrome P450 variants are functional.

As amended, claims 146-153 are directed to functional cytochrome P450 variants. Exemplary functional amino acid sequences, *e.g.*, SEQ ID NOS:11-13, are shown in Example 8 (pp. 51-60) of the specification. Reconsideration and withdrawal of this rejection is therefore respectfully requested.

Claim 154 has been rejected as allegedly indefinite for being directed to, in the Examiner's opinion, a "variant of a variant."

It is respectfully submitted that this rejection is in error. Claim 154 is directed to a function-conservative variant, having specific amino acid substitutions at specific amino acids of SEQ ID NO:2. The term "function-conservative" is well-known

in the art, and is also defined at page 21, lines 1-7 of the specification. Nevertheless, to advance prosecution, claim 154 has been amended to be directed to the P450 variant of 152 comprising at least one mutation selected from arginine or histidine at position 331 or isoleucine, methionine, or valine at position 280, of SEQ ID NO:2.

It is respectfully submitted that this rejection has thereby been overcome and should be withdrawn.

Claims 161, 164, and 167, and claims dependent thereon, have been rejected as allegedly indefinite. Specifically, the Examiner contends that it is not clear how it can be concluded that a variant is 10 times more active or stable than the wild-type, or how one could identify variants or mutants with specific amino acid changes based on activity assays.

The Examiner's attention is respectfully directed to MPEP 2106, section A2, which recites:

However, the applicant need not explicitly recite in the claims every feature of the invention. For example, if an applicant indicates that the invention is a particular computer, the claims do not have to recite every element or feature of the computer. In fact, it is preferable for claims to be drafted in a form that emphasizes what the applicant has invented (*i.e.*, what is new rather than old). *In re Dossel*, 115 F.3d 942, 946, 42 USPQ2d 1881, 1884 (Fed. Cir. 1997).

Claims 161, 164, and 167 are directed to cytochrome P450 oxygenase variants having specific properties (*e.g.*, at least 10 times the activity of the wild-type

enzyme). It is not necessary, however, for the claims to recite how each property can be assessed and identified. One of ordinary skill in the art can easily envision multiple assays suitable for comparing the oxygenase activity or stability of the enzyme variants to that of the corresponding wild-type enzyme, especially since several such assays are provided by the specification and since the claims recite that the oxygenase variant has at least 90% sequence identity to SEQ ID NO:2. See specification, page 21, lines 7-15; page 22, line 16 to page 27, line 27, and the Examples, pages 28-62.

However, to advance prosecution, claims 161, 164, and 167 have been amended to recite a step of selecting any test enzyme having the specified properties. Accordingly, it is submitted that the rejection should be withdrawn.

Rejections Under 35 U.S.C. 112, 1st Paragraph

Claims 146-159 and 161-166 have been rejected as allegedly not being enabled by the specification. Specifically, the Examiner admits that the specification is probably enabling for variant cytochrome P450 which are highly similar to P450_{cam}, exemplifying 95-99% sequence homology to SEQ ID NO:2, but contends that the specification is not enabling for variants that are not structurally related to cytochrome P450 with such high structural homology. According to the Examiner, the specification does not teach how one skilled in the art would go about finding out or making amino acid changes *corresponding to* amino acids 242, 280, or 331 in cytochrome P450 (Office Action, page 5; emphasis in original).

It is respectfully submitted that the amended claims are fully enabled by the specification. All claims now recite that the claimed cytochrome P450 variants:

- (1) are functional, *i.e.*, have oxygenase activity, or have a specific functional characteristics as compared to *P. putida* cytochrome P450 oxygenase; and
- (2) have at least 90% sequence identity to SEQ ID NO: 2.

In addition, all claims recite at least one further characteristic of the novel cytochrome P450 variants. Specifically, claims 146-155 and 167-177 provide the positions of the mutations, as follows: mutations at positions corresponding to specific residues of SEQ ID NO:2 (claims 146-152, 167-169), mutations in SEQ ID NO:2 itself (claims 153 and new claims 170-177), and function-conservative or hybridizing sequences thereof claims 154-155), whereas claims 156-166 provide specific characteristics as to the function of the variants as compared to that of SEQ ID NO:2. Amended claims 146-151, 153, and 154 also recite variants having a mutation in a glutamic acid, arginine, or cysteine residue at a position corresponding to position 331, 280, or 242, respectively, of SEQ ID NO:2.

Taken together, the pending claims describe and enable cytochrome P450 variants that might require some experimentation, but no undue or unreasonable experimentation, for one of skill in the art.

"To be enabling, the specification of the patent must teach those skilled in the art how to make and use the full scope of the claims of invention without 'undue experimentation.' " Genentech Inc. v. Novo Nordisk, A/S, 42 USPQ 2d 1001,

1004 (Fed. Cir. 1997) (quoting *In re Wright*, 27 USPQ 2d 1510, 1513 (Fed. Cir. 1993)). The court has held that a patent specification complies with the statute even if a "reasonable" amount of routine experimentation is required but such experimentation must not be "undue". *Enzo Biochem Inc. v. Calgene, Inc.* 52 USPQ 2d 1129, 1135 (Fed. Cir. 1999) (citing *In re Wands*, 8 USPQ 2d at 1404). The court in *Enzo Biochem* looked favorably on the factors set forth in *In re Wands* to consider in determining whether disclosure requires undue experimentation, which are:

- (1) the quantity of experimentation necessary,
- (2) the amount of direction of guidance presented,
- (3) the presence or absence of working examples,
- (4) the nature of the invention,
- (5) the state of the prior art,
- (6) the relative skill of those in the art,
- (7) the predictability or unpredictability of the art, and
- (8) the breadth of the claims.

Enzo Biochem, 52 USPQ 2d at 1135-1136 (quoting *In re Wands*, 8 USPQ 2d at 1404).

Applying the Wands factors to the facts set forth above to the amended claims shows that the specification enables the claimed invention.

First, the quantity of experimentation necessary to prepare the claimed cytochrome P450 oxygenase variants is not great, and by no means undue. As

outlined on pp. 17-19 of the previous Response to Official Action, filed March 8, 2002, hereby incorporated by reference, sequence alignment using SEQ ID NO:2 as a reference is facilitated by making reference to a number of sequence alignment and sequence identity determination programs, as well as sequence databases such as GenBank, enabling searching for and aligning a vast number of sequences in a short period of time. This guidance to use standard techniques in aligning polypeptides with a reference sequence is sufficient to identify and enable the claimed enzyme variants. *See*, specification at page 21, lines 7-15. Determining the amino acids of a first polypeptide sequence which correspond to amino acids in a second polypeptide sequence is a fundamental feature of sequence identity determination, well known to those of skill in the art.

Also, while the experimentation associated with screening for oxygenase activity might require some labor, such screening is merely routine lab work, by no means undue. Notably, the experimental work upon which the present invention is partly based involved the screening of libraries of hundreds of thousands of mutants (see page 53, line 27 to page 54, line 14), thus establishing that screening of vast numbers of oxygenase variants is disclosed, enabled, and is a routine procedure, by no means burdensome, especially when armed with the present disclosure.

Second, as for the amount of guidance given, the specification provides a wild-type cytochrome P450 sequence (SEQ ID NO:2) and three exemplary variant sequences (SEQ ID NO:11-13) that are at least 90% identical to SEQ ID NO:2,

primers, as well as sequence alignment programs, and detailed descriptions of methods to prepare variants and screen them for oxygenase activity.

Third, the present application reports working examples preparing and screening for variants of a cytochrome P450 wild-type sequence. Three preferred variants were identified, having up to 2 mutations in a protein sequence of 414 residues and being at least 90% identical to SEQ ID NO:2. Specifically, Example 8 describes the selection process for these variants, establishing their relative rates of oxygenase activity as compared to *P. putida* cytochrome P450, and the sequencing of the same. It is submitted that these working examples are sufficient to enable the full scope of the claimed invention.

Fourth, the nature of the invention is, contrary to the Examiner's assertions, one of a fairly routine and standard experimental systems. Improving enzymes by mutation of certain residues, and the methods to do so, are standard in the art and recognized as routine experimentation.

Fifth, the state of the prior art is highly advanced. The enclosed excerpt from Gribskov and Devereaux's "Sequence Analysis Primer" (Freeman and Co., 1992), attached hereto as Exhibit A, clearly establish that alignment and sequence identity determination of sequences were routine a decade ago, and Peterson et al. (In: Cytochrome P450: Structure, Mechanism, and Biochemistry. 2nd Ed., edited by Ortiz de Montellano, PR. Plenum Press, New York, 1995), attached hereto as Exhibit B, show that structure-function relationships of cytochrome P450 oxygenases were well-

known in the art prior to the filing of the application. Notably, Peterson show alignment of 18 cytochrome P450 oxygenases in Figure 11 (Peterson, pp. 163-165), depicting the determination of which amino acids correspond to which in a reference cytochrome P450 oxygenase such as P450_{cam}. Nothing provided by the Examiner establishes with any requisite particularity a contrary conclusion, *i.e.*, that the state of the art would be less than advanced.

With respect to item six, the relative skill of those in the art paralleled the advanced state of the art. Thus, the skilled artisan would not have any problem generating or identifying the claimed enzyme variants.

With respect to item seven, the predictability of the preparing and identifying cytochrome P450 variants is represented by the Examples, showing successful generation and evaluation of cytochrome P450_{cam} variants. Conducting a mere sequence alignment to establish the sequence identity of the variant to SEQ ID NO:2 and evaluating the aligned residues is routine, and has been conducted by those of skill in the art for many years. For example, Szklarz et al. (J Biomol Struct Dyn 1994;12:61-78), attached hereto as Exhibit C, used alignment of cytochrome P450 2B1 with P450_{cam} to interpret the effect of site-directed mutations of P450 2B1. Moreover, the Szklarz reference showed that there was a high degree of predictability even in 1994, 5 years prior to the priority date of this application. Even though an absolute predictability in assessing the effect of a residue may not be achievable based on modeling or alignment alone, Szklarz noted that any result indicated by his

alignment model could be readily tested or verified experimentally ("The model indicates other residues likely to be important for enzyme function, such as Tyr-111 ..., which can be tested experimentally"; Szklarz, Abstract). Naturally, the field has also progressed since the publication of this reference.

Finally, the full scope of each claim is fully enabled. The specification provides functional cytochrome P450 variants having a sequence identity of at least 90% to SEQ ID NO:2, displaying mutations at sites corresponding to specific SEQ ID NO:2 residues and/or having a novel level of oxygenase activity or stability as compared to SEQ ID NO:2. This is not undue scope or breadth, particularly when considering that the fundamental function of, *i.e.*, reaction catalyzed by, cytochrome P450 oxygenases has been known for decades (see, reaction scheme on page 151 of Exhibit B, referring to a reference published in 1980), and cytochrome P450 oxygenases displaying as low as 7% sequence identity are "drastically similar" in their overall structure (Peterson, Exhibit B, p. 156, 3rd paragraph). Accordingly, since cytochrome P450 enzymes are characterized by close structural and functional relationships, a 90% sequence identity easily provides full enablement of the claimed invention.

Thus, since the claims are fully enabled by the specification according to the *In re Wands* factors, it appears, then, that the Examiner could instead be

concerned that some of the claims may read on inoperative embodiments. Any such concern is, however, does not offend §112, due to the following reasons.

...typically, inoperative embodiments are excluded by the language in a claim (*e.g.*, preamble)...MPEP 2164.08(b).

The amended claims are all directed to functional oxygenase variants. Specifically, amended claims 146-159 and 167-169, as well as new claims 170-177, are directed to functional cytochrome P450 variants, and the oxygenase variants of claims 160-166 all include features or steps describing oxygenase activity.

Furthermore, as outlined herein, the high-throughput screening methods provided by the specification, as well as the detailed knowledge of the structural and functional features of cytochrome P450 oxygenases (see, *e.g.*, Exhibit B, especially section 4.4), provides for full enablement of the claimed oxygenase variants, without any undue experimentation.

In view of the foregoing amendments and remarks, it is submitted that the claims meet the enablement requirement, and the Examiner's rejection should be withdrawn.

The Examiner has also rejected claims 146-166 for allegedly not complying with the written description requirement. Specifically, the Examiner contends that no information beyond the characterization of a single species of the genus of polypeptides encompassed by the claims is provided.

Applicants respectfully submit that the Examiner's contention is incorrect.

The specification provides a multitude of cytochrome P450 variants having improved oxygenase activity. For example, Figure 19 and 20 show improved P450 activity of a vast number of mutants, many of which have at least twice the activity of P450_{cam} (note activity of wild-type indicated as "WT" in the figures). Three preferred mutants were further characterized by sequencing, thus providing detailed structural characteristics for three specific variants in addition to the functional characteristics (see, *e.g.*, Table 2, page 54, and page 55).

In the Office Action, the Examiner refers to the revised Guidelines concerning compliance with the written description requirement. Attached hereto as Exhibit D is an excerpt of these Guidelines, describing an example (Example 14) that is similar to the functional cytochrome P450 oxygenase variants of the invention. Specifically, the claim presented in the Example reads:

A protein having SEQ ID NO:3 and variants thereof that are at least 95% identical to SEQ ID NO:3 and catalyze the reaction $A \rightarrow B$.

In the subsequent analysis, it is stated that:

[T]he procedures for making variants of SEQ ID NO:3 are conventional in the art and an assay is described which will identify other proteins having the claimed activity. Moreover, procedures for making variants of SEQ ID NO:3 which have 95% identity to SEQ ID NO:3 and retain its activity are conventional in the art... The single species disclosed is representative of its genus because all members have at least 95% structural identity with the reference compound and because of the presence of an assay which applicant provided for identifying all of the at least 95%

identical variants of SEQ ID NO:3 which are capable of the specified catalytic activity.

In the Example, it was concluded that the claim met the requirements for written description, even though no variant of SEQ ID NO:3 was presented in the "hypothetical" specification.

The pending claims are similar to this Example from the Guideline, reciting both polypeptide function, sequence, and percentage sequence identity. The present claims also recite specific positions for the amino acid mutations and/or activity comparisons to that of reference polypeptides.

In the context of the present invention, it is clear that cytochrome P450 enzymes is a particularly well-characterized protein family whose close functional and structural relationships allows for a broad (*e.g.*, 90-100%) range of sequence identity. For example, Peterson et al. (Exhibit B) reported striking structural similarities between cytochrome P450 oxygenases sharing only 7% sequence identity (p. 156), and concluded that they believed that all members of the P450 gene superfamily would have fundamentally the same secondary structural elements. Notably, the specification enables, describes, and supports 90% sequence identity (see specification, *e.g.*, page 21, lines 7-15), and the Examiner has not provided any evidence that 90% is not described or would be less enabled. The Examiner acknowledges, apparently from personal knowledge, that the 95-99% sequence homology recited in the Office Action would be appropriate for enablement purposes

(Office Action, page 5), but this specific case, the specification provides at least 90% (*i.e.*, 90%-100%) sequence identity.

Furthermore, as described above, the specification describes not only the wild-type sequence but also three variant sequences as opposed to the single sequence exemplified in the hypothetical example in the Guidelines. Particular embodiments recited in the claims also set forth specific residues for mutation, as well as specific protein sequences. For example, claims 146-151 calls for a mutation in a glutamic acid, arginine, or cysteine residue corresponding to amino acids 331, 280, and 242 of SEQ ID NO:2, respectively, and new claims 170-177 are directed to specific SEQ ID NO:2 variants.

Accordingly, as described and discussed in detail above, the claims set forth cytochrome P450 mutants characterized by functional as well as structural characteristics which fully complies with the written description requirement. Therefore, it is respectfully submitted that this rejection should be reconsidered and withdrawn.

Rejection Under 35 U.S.C. § 102

The Examiner maintains that claim 155-159 are anticipated by either one of Manchester et al. (a) (Protein Engineering 1995;8:801-807) and Manchester et al (b) (Biochemie 1996;78:714-722). Specifically, the Examiner has taken the position that characteristics such as having an oxygenase activity or stability that is 2-10 times

that of the wild-type enzyme are inherent of Manchester's variant and hence read upon the claims, and that Manchester did not specifically state that the oxygenase activity was lost in their mutant.

It is respectfully submitted that the Examiner's position is contrary to the evidence accumulated in the art regarding enzyme mutants. As shown by Arnold et al. (Curr Opin Chem Biol 1999;3:54-59, attached hereto as Exhibit E) and Arnold (Acc Chem Res 1998;31:125-131; attached hereto as Exhibit F), one activity often come "at the expense of another." For example, as shown by Arnold et al. (page 54, 2nd column, 3rd paragraph), "[f]our rounds of shuffling increased the activity of aspartate aminotransferase for valine and 2-oxovaline by five orders of magnitude, while decreasing by 30-fold the activity towards the natural substrate, aspartate." Similarly, in the case of another enzyme, Arnold (page 126, 2nd column, 1st paragraph) described that "[d]irected evolution of a loracarbef pNB esterase, however, came at the cost of the enzyme's activity toward a smaller substrate, *p*-nitrophenyl acetate, in low concentrations of DMF. Importantly, the phenomenon was summarized as follows (Arnold, *Id.*):

It is often seen that while one particular feature evolves, other properties will drift. If however, two properties are coupled to one another, the evolution of one can have a dramatic effect on the other.

In the case Manchester, there is no disclosure and can be no presumption of coupling. Dehalogenation of a substrate in the absence of oxygen cannot

reasonably be coupled to oxygenation in the presence of an oxygen donor. Hence, scientific data supports that enhancing dehalogenation would more than likely come at the expense of the oxygenase activity of Manchester's mutant. Since Manchester did not show that oxygenase activity was retained, much less increased by a factor of 2-10, this reference simply cannot anticipate the claimed invention.

Likewise, new claims 170-189 are novel and unobvious over Manchester, since these claims are directed to cytochrome P450 variants which have mutations at novel sites, or have improved activity or stability in the presence of a peroxide as an oxygen donor. Manchester is completely silent on these issues.

Unstated properties, especially in the case of improved properties, cannot be presumed inherent in a reference (MPEP 2112, "[t]he fact that a certain result or characteristic *may* occur or be present in the prior art is not sufficient to establish the inherency of that result or characteristic". *In re Rijckaert*, 9 F.3d 1531, 1534, 28 USPQ2d 1955,1957 (Fed. Cir. 1993), (emphasis added)), nor can the Examiner rely on personal knowledge or belief unsupported by evidence, or on hindsight from the invention.

Accordingly, for all of these reasons, reconsideration and withdrawal of this rejection is earnestly requested.

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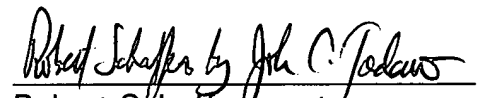
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Therefore, in view of the above amendments and remarks, it is respectfully requested that the application be reconsidered and that all pending claims be allowed and the case passed to issue.

If there are any other issues remaining which the Examiner believes could be resolved through either a Supplemental Response or an Examiner's Amendment, the Examiner is respectfully requested to contact the undersigned at the telephone number indicated below.

Respectfully submitted,


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EXHIBITS:

- A: Similarity and Homology, In: "Sequence Analysis Primer", Gribskov and Devereaux (Eds.), Freeman and Co., 1992, pp. 89-157.
- B: Peterson et al., In: Cytochrome P450: Structure, Mechanism, and Biochemistry. 2nd Ed., edited by Ortiz de Montellano, PR. Plenum Press, New York, 1995.
- C: Szklarz et al., J Biomol Struct Dyn 1994;12:61-78.
- D: Excerpt from the Revised Written Description Guidelines, pp. 53-55.
- E: Arnold et al., Curr Opin Chem Biol 1999;3:54-59.
- F: Arnold, Acc Chem Res 1998;31:125-131.

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